MICROBAC®

Microbac Protocol

AOAC Germicidal Spray Test

Healthcare - Confirmatory

Testing Facility
Microbac Laboratories, Inc.
105 Carpenter Drive
Sterling, VA 20164

Prepared for
Virox Technologies, Inc.
2770 Coventry Road
Oakville, Ontario
L6H 6R1

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Microbac Protocol: 506.1.05.13.20

Microbac Project No.: 506 - 217

OBJECTIVE:

This test is designed to prove germicidal effectiveness label claims for products registered with the Environmental Protection Agency and Canada (if applicable) as spray germicides. It evaluates the effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces. The test is based on the Official Methods of Analysis (AOAC 961.02) (2012) and is required by EPA Product Performance Guidelines. This test meets the EPA OCSPP 810.2000 and 810.2200 Product Performance Test Guidelines (February 2018), the Series 810 2019 Frequently Asked Questions (FAQ) document (August 28, 2019), and Health Canada "2014 Guidance Document – Safety and Efficacy Requirements for Hard Surface Disinfectant Drugs" as applicable.

TESTING CONDITIONS:

Ten replicates will be evaluated using a two lots of a single test substance. Glass carriers inoculated with *Staphylococcus aureus* and *Pseudomonas aeruginosa*, will be sprayed for the specified time and distance directed by the sponsor or label instructions and transferred into individual tubes containing neutralizing recovery broth.

MATERIALS:

A. Test, control and reference substances will be supplied by the sponsor of the study (see last page). As per CFR 40.160.105:

- The identity, strength, purity, and composition, or other characteristics which will appropriately define the test, control, or reference substance shall be determined for each lot and shall be documented by the sponsor before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control, or reference substance shall be documented and retained by the sponsor.

- When relevant to the conduct of the study the solubility of each test, control, or reference substance shall be determined by the sponsor before the experimental start date. The stability of the test, control, or reference substance shall be determined by the sponsor before the experimental start date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each lot.

The test substance will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test substance such as dilution or specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures Microbac Laboratories, Inc. (Microbac) testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

Microbac will retain all unused test substances for a period of at least one year after completion of the test, and then discard them in a manner that meets the approval of the safety officer.

B. Materials supplied by Microbac, including, but not limited to:

1. Challenge microorganisms, required by the sponsor of the study:
   a. *Staphylococcus aureus*, ATCC 6538
   b. *Pseudomonas aeruginosa*, ATCC 15442

2. Media and reagents:
   a. Synthetic Broth (SB)
   b. Nutrient Broth (NB)
   c. Neutralizer: Recovery broth with required neutralizer(s)
   d. Letheen Broth (LB)
   e. Heat-inactivated fetal bovine serum (if required)
   f. Phosphate Buffered Dilution Water (PBDW)
   g. Tryptic Soy Agar (TSA)
   h. Mannitol Salt Agar (MSA)
   i. MacConkey Agar (MCA)

3. Laboratory equipment and supplies, including glass microscope slides (1" x 3" with a 1" x 1" surface for contamination and treatment)

TEST SYSTEM IDENTIFICATION:

All test and control tube racks will be labeled with microorganism, test substance (if applicable) and project number prior to initiation of the study and during incubation. Petri dishes will be labeled with microorganism prior to initiation of the study and microorganism and project number during incubation. Test substance and usage will be traced according to SOPs extant in the laboratory.

EXPERIMENTAL DESIGN:

A. Inocula preparation:

A single frozen cryovial of stock culture will be defrosted at room temperature and then briefly vortexed to mix. A 10 µL aliquot of the thawed stock will be added to a tube containing 10 mL of SB (NB for *Pseudomonas aeruginosa*). The tubes will be vortex mixed and incubated at 36±1°C for 24±2 hours. Daily transfers will be made for at least one but no more than five consecutive days.

For the final subculture transfer, tubes containing 10 mL SB (NB for *Pseudomonas aeruginosa*) will be inoculated with 10 µL of culture per tube and incubated at 36±1°C. After 48-54 hours, cultures will be used for contaminating the carriers.

The pellicle formed in the *Pseudomonas aeruginosa* culture will be removed prior to carrier contamination by gently aspirating the pellicle away from the broth using a pipette or by vacuum removal. Care will be taken to avoid harvesting the pellicle from the bottom of the tube. The culture will be visibly inspected for pellicle fragments. If pellicle fragments are present, the culture will not be used for testing.

Each tube of inoculum will be agitated on a Vortex-type mixer for 3-4 seconds, and then allowed to sit for 10 minutes. The upper portion of each culture will be removed, leaving any debris or clumps and transferred to a sterile flask and pooled.

If requested by the sponsor, heat-inactivated fetal bovine serum will be added to the culture to achieve an organic load of 5%.

B. Carrier preparation and inoculation:

The new carriers will be visually screened and discarded if visibly damaged (scratched, chipped or nicked). The carriers will be rinsed with 95% ethanol followed by a rinse with deionized water to remove oil and film on the slides. The carriers will be sterilized by placing in evaporating dishes matted with two pieces of filter paper, heating them in a hot air oven for two hours at 180C, cooling and storing them at room temperature until use.

Using a positive displacement pipet, a 0.01 mL (10 µL) aliquot of each culture will be transferred onto a one-square inch area on the sterile carriers (in Petri dishes) and immediately spread uniformly over the entire area with a sterile glass rod. Each dish will be covered promptly and the operation will be repeated for the rest of the carriers, for each microorganism. Carriers will be dried for 30-40 minutes at 36±1°C. The humidity level of the incubator during the drying phase required for the inoculated carriers will be monitored and reported.

C. Test substance preparation:

The test substance will be prepared and applied exactly as directed by the sponsor of the study. If mixing of components or dilution is required, the prepared test substance will be used within three hours for testing.

D. Test:

Note: The temperature and humidity level of the laboratory during the test phase will be monitored and reported.

Ten carriers per organism will be sprayed in a horizontal position until thoroughly wet from 6" – 8". Each carrier will be held in a horizontal position for the exposure time as specified by the sponsor. After the contact period, the excess liquid will be allowed to drain from the carrier and the carrier will be transferred to a tube containing 20 mL of Neutralizer and shaken thoroughly. For products with ≤ 1-minute contact time, the transfer will be made within ±3 seconds.

All subculture tubes containing the carriers will be incubated for 48±2 hours at 36±1°C. All observations will be recorded as growth or no growth.

E. Controls:

1. Sterility controls:

One sterile carrier will be added to a tube of Neutralizer and incubated with the test in order to demonstrate the sterility of the media used in the study.

2. Viability controls:

For each challenge microorganism, two inoculated carriers will be independently transferred into tubes of Neutralizer and incubated with the test to serve as comparison for the test cultures.

3. Neutralizer effectiveness:

For each challenge microorganism, per lot, two sterile, non-inoculated carriers will be exposed to the test substance for the contact time evaluated, and then transferred into individual tubes of Neutralizer. To each tube, fewer than 100 colony forming units (CFU) of the challenge microorganism will be added and the count of the bacteria inoculated into these tubes will be confirmed in duplicate TSA pour plates. The tubes and plates will be incubated with the test.

4. Carrier counts:

For each challenge microorganism, per lot, three inoculated and dried carriers will be randomly selected for carrier counts immediately (single set of two) before the testing and immediately (single set of two) after testing.

Dried inoculated carriers will be placed individually into tubes containing 20 mL LB. The tubes will be immediately vortex mixed for 120±5 seconds (60±5 seconds for *Pseudomonas aeruginosa*). After vortex mixing, serial ten-fold dilutions of each suspension will be performed in PBDW blanks. Duplicate one mL aliquots from selected dilutions will be plated in TSA pour plates. Diluting and plating will be completed within 2 hours after vortex mixing. All plates will be incubated with the test and the average CFU/carrier determined.

5. Confirmation of challenge microorganism:

   All the viability controls and at least 20% of the test tubes showing growth will be streaked onto TSA plates (if three or less test tubes are positive within a group of 10 replicates, all positive tubes will be streaked onto TSA). The same tubes will also be streak onto the corresponding specialty media: MSA (for *Staphylococcus aureus*), MCA (for *Pseudomonas aeruginosa*). All plates will be incubated for 24±2 hours at 36±1°C. Gram stains will be performed from these streaks to confirm growth of the challenge microorganism.

PRODUCT EVALUATION CRITERIA:

According to the EPA, the test substance passes the test if no visible growth is observed in ≤ 1 replicate out of 10 for each lot. All controls must meet the test acceptance criteria. There is no statistical method proposed for this protocol.

CALCULATIONS:

- The $\log_{10}$ density (LD) for each carrier will be determined based on the following:
  - Dilutions yielding counts to 300 CFU will be used.
  - Plate counts of 0 will be included in the calculations.
  - The CFU/mL (of broth) will be calculated:

$$\text{CFU/mL} = \frac{(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})}{10^{-x} + 10^{-y} + 10^{-z}}$$

Where 10^{-x}, 10^{-y}, 10^{-z} are the dilutions plated

  - The CFU/carrier will be calculated by multiplying the CFU/mL by the volume of broth into which the challenge microorganism was harvested from the carrier by vortex-mixing (20 mL).
  - The LD for each carrier will be calculated by taking the Log_{10} of the density (per carrier).
  - There is no statistical analysis for this test

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- Based on the AOAC method, the geometric mean of the carrier counts must be between 1.0 x 10^5 and 3.2 x 10^6 CFU/carrier for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, the geometric mean may fall outside this range and the test is still acceptable for evaluation if the geometric mean of the carrier counts are:
  - For average counts that are below the stipulated range, the test must be repeated if the performance standard is achieved.
  - For average counts that are below the stipulated range, the test does not need to be repeated if the performance standard is not achieved.
  - For average counts that are above the stipulated range, the test does not need to be repeated if the performance standard is achieved.
  - For average counts that are above the stipulated range, the test must be repeated if the performance standard is not achieved.
- The recovery broth with neutralizers must be proven effective
- The sterility controls must be negative for growth
- The viability control must be positive for growth.
- The purity of the challenge microorganism must be confirmed based on the procedures employed for confirmation.
- Regarding any presence of contamination in subculture media:
  - Contaminants are defined as microorganisms which are not the test organism that are present in the subculture media.
  - Methods such as Gram staining, colony morphology and biochemical assays may be used to identify the contaminant. The result will be reported to the EPA.
  - In the case where a contaminant and the test organism are both present in the subculture media, the outcome will be considered a positive carrier.
  - For a 10-carrier test: A test with one or more contaminated carrier(s) is invalid and may be repeated one time using another 10-carrier test.

DATA PRESENTATION:

The final report will include the following information:

- The number of positive carriers.
- The average colony-forming units per carrier.
- The results of all controls.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at Microbac Laboratories, Inc., 105 Carpenter Drive, Sterling, VA 20164.

REGULATORY COMPLIANCE AND QUALITY ASSURANCE (GLP studies only):

This study will be performed in compliance with the US Environmental Protection Agency's Good Laboratory Practices (GLP) regulations, 40 CFR 160 (note: information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test substance resides with the sponsor of the study unless otherwise stated).

The Quality Assurance Unit of Microbac will inspect the conduct of the study for GLP compliance. The dates of the inspections and the dates that findings are reported to the study management and study director will be included in the final report.

PROTOCOL AMENDMENTS AND DEVIATIONS:

Any protocol amendment(s) and protocol deviation(s) identified will be reported in project sheet(s) and included in the final report.

REPORT FORMAT:

The report will contain all items required by EPA 810.2200 and be compliant with EPA PR Notice 2011-3 (replaced PRN 86-5). Microbac employs a standard report format for each test design. Each final report will provide at least the following information:

- Sponsor identification
- Test substance identification
- Type of assay and project number
- Study start and end time (clock time)
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria, if applicable
- Dates of study initiation and completion (GLP studies only)
- Signed Quality Assurance and Compliance Statements (GLP studies only)

RECORDS TO BE MAINTAINED:

For all GLP studies, the original signed final report will be sent to the Sponsor.

A draft report will be provided to Sponsor for review prior to finalization of the report. All raw data, protocol, protocol modifications, test substance records, copy of final report, and correspondence between Microbac and the sponsor will be stored in the archives at Microbac Laboratories, Inc., 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test substance; challenge microorganism used, and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs the protocol will be the initiation date. All project sheets issued will be forwarded to the study sponsor for appropriate action.

REFERENCES

1. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides, Guidance for Efficacy Testing, February 2018.

2. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, Frequently Asked Questions (FAQ) for OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces - Guidance for Efficacy Testing, 2019.

3. *Official Methods of Analysis of the AOAC International*, Chapter 6, Disinfectants, Official Method 961.02, Germicidal Spray Products as Disinfectants, Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417

4. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces - Guidance for Efficacy Testing, 2019.

5. U.S. Environmental Protection Agency, Office of Pesticide Programs, Microbiology Laboratory, Environmental Science Center, Ft. Meade, MD, Standard Operating Procedure for Germicidal Spray Products as Disinfectants (GSPT): Testing of *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella enterica*. SOP Number: MB-06-09. Date Revised: 09-29-17.

MISCELLANEOUS INFORMATION: The following information is to be completed by sponsor before initiation of study:

A. Test substance information:

Test Substance Name	Oxyteam (Accel Concentrate)		
Active ingredient(s)	H2O2		
Lot No.	Lot No. 1	Lot No. 2	Lot No. 3
	14476	14477	
Dilution	☑ 1: 16 (1 part test substance + 16 parts diluent) ☐ Ready to Use ☐ Other: _____		
Diluent	☐ Not applicable (test substance is Ready to Use) ☐ _____ ppm ± 2.9% AOAC Hard Water ☑ Other: Unsofted tap water 180-210 ppm calcium carbonate		
Concentration	☑ Lower Certified Limit (LCL) ☐ At or below nominal		

B. Test Conditions:

Contact time	5 minutes (must be ≤ 10 minutes
Contact temperature	Ambient Room Temperature (20±1C)
Test Substance Application	Spray until thoroughly wet from a distance of 6" – 8"

C. Organic load – serum (HI FBS) added to achieve 5% in the inoculum: ☑ yes ☐ no

D. Precautions/storage conditions: MSDS and/or C of A provided: ☑ yes ☐ no

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REPORT HANDLING AND STUDY CONDUCT: ☑ EPA ☑ Health Canada, GLP

PROTOCOL APPROVAL:

Sponsor Signature: _____ Date: 5-15-2020

Sponsor Name: Faraz Alderson

Study Director Signature: _____ Date: 5/29/20

Study Director Name (Print): Travis R. Farley